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USE OF THE ACID DYE TECHNIQUE FOR QUANTITATION OF PHARMACEUTICAL AMINES ELUTED FROM THIN-LAYER CHROMATOGRAMS

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SUMMARY

A quantitative thin-layer chromatographic procedure coupled with the acid dye technique for the estimation of the amount of eluted sample is described for the analysis of mixtures of pharmaceutical amines. With the five amines studied, the over-all recovery after application, development, adsorbent removal, elution and color development was 97.1% with an average coefficient of variation of 0.7% (based on ten chromatoplates having six replicates on each). Analysis of three binary mixtures of amines by two operators showed no appreciable difference between the two, with average assay values for each of 100.6% and 100.1%.

INTRODUCTION

The acid dye technique is a general procedure for the quantitative analysis of organic amines, with many modifications having been described[1]. The most common involves extraction of the ion-pair formed between the amine and an indicator dye from an aqueous buffered system into an organic phase and spectrophotometric measurement of the organic layer. The method has been shown to be rapid, accurate and precise, and to afford a relatively high and similar sensitivity for many amines regardless of other functional or chromophoric groups present[2,3]. Neutral, acidic or weakly basic materials as well as most excipients do not interfere.

Many pharmaceutical preparations contain mixtures of amines which often require separation prior to determination of the individual components. Thin-layer chromatography offers a rapid means for the separation, hence a combination of this procedure with the acid dye technique was investigated as a general method for the analysis of mixtures of amines. In addition, the results of the study serve to demonstrate the precision and accuracy that may be obtained with quantitative thin-layer chromatography.

EXPERIMENTAL

Reagents and materials

Thin-layer chromatography. Chromatoplates, 20 × 20 cm were coated to a thickness of 250 μ using a slurry of 35 g of Silica Gel DSF-5 (Camag) with 67 ml of water for five plates. The developing solvent used was chloroform–methanol–conc. ammonia (100:8:1). In this solvent system, the approximate R_F values for ephedrine, cyclizine, propoxyphene, chlorpheniramine and diphenhydramine were 0.16, 0.62, 0.66, 0.67 and 0.82, respectively. Detection was by short-wave UV light.

Acid dye technique. A buffer solution of pH 8 (McIlvaine) was prepared by mixing 97.3 ml 0.2 *M* Na_2HPO_4 with 2.7 ml 0.1 *M* citric acid. For the Bromthymol Blue (BTB) buffer solution sufficient Bromthymol Blue was dissolved in buffer of pH 8 to give a molar ratio of dye to drug of 10–13:1 when using 5 ml of BTB buffer solution and 5 ml of sample solution. The BTB buffer solution (250 ml) was shaken with 50 ml of photometric solvent before use. The photometric solvent consisted of benzene (AR) or benzene (AR) containing 1% by volume of isoamyl alcohol (AR).

Reference solutions. Methanolic solutions of chlorpheniramine maleate, cyclizine monohydrochloride, diphenhydramine hydrochloride, ephedrine sulfate and propoxyphene hydrochloride having concentrations in the order of 6–8, 9, 8, 10 and 12.5 μg/μl, respectively.

Sample solutions of mixtures. Methanolic solutions of each two-component mixture were prepared in the concentrations stated above for the reference solutions.

Procedure

Spotting the sample. An Agla micrometer syringe was filled and clamped securely in a vertical position. The chromatoplate was raised mechanically so that the needle tip pierced the silica gel layer and touched the backing glass plate. The syringe needle had been blunted by careful filing so as to allow the liquid to flow smoothly from the syringe onto the silica gel layer. Ten microliters of sample solution were applied in one operation by smoothly turning the micrometer head.

Chromatography and recovery of sample. Chromatography was carried out in filter paper lined jars and the solvent was allowed to travel 15 cm (about 45 min). The plates were air dried and sample spots located under short-wave UV light. A sharp stylus was used to outline the sample spot about 2 mm from the outer edge. Each sample spot was removed from the plate using a modified medium porosity sintered glass funnel of 10 mm diameter. The outlet end was drawn out to provide a tip about 3.5 cm in length and 1 mm I.D. The upper end was joined about 1.5 cm above the sintered glass disc to a 7 cm length of 4 mm O.D. glass tubing bent at 45° at the midway point. Suction was applied to the outlet end, and the inlet end (having a short length of tygon tubing projecting 0.5 cm beyond the end) applied to the plate for removal of the silica gel layer. For the blank determination, an area similar to the sample was removed.

Elution of the sample and quantitative determination. Following removal of the sample spot, a solvent reservoir (glass tubing 8 mm I.D. × 12 cm with an outlet of 4 mm O.D. tubing) was connected through the tygon tubing to the upper end of the sintered glass funnel. The outlet end of the funnel was then inserted through a rubber

stopper into a centrifuge tube*. Gentle suction was provided by inserting a syringe needle (connected to vacuum) through the rubber stopper into the centrifuge tube. For a number of simultaneous determinations, several of these assemblies were connected to a common vacuum manifold system using syringe needles and tygon tubing for connecting linkages.

The sample was eluted with 5 ml of 1% (v/v) HCl flowing from the reservoir through the silica gel into the centrifuge tube. The amount of vacuum was adjusted to give an elution time of about 10 min. To the eluate were added 5 ml of buffer solution, 5 ml of BTB buffer solution and 10 ml of photometric solvent. The tube was shaken vigorously for 1 min (alternatively, a mechanical device which tumbled 15 tubes end-over-end at 100 r.p.m. for 5 min was found convenient for processing a number of tubes simultaneously). The tubes were centrifuged for 2 min and the supernatant decanted carefully into a clean dry cuvette for measurement of absorbance at 410 mμ against a blank prepared similarly from silica gel removed from the plate.

RESULTS AND DISCUSSION

Experimental conditions for the acid dye technique were such that approximately 75–100 μg of drug in the 5 ml of eluate gave a satisfactory absorbance at 410 mμ when partitioned with BTB from the buffered aqueous phase into 10 ml of photometric solvent. Each drug examined followed Beer's Law over the concentration range of interest, as shown in Table I. Benzene containing 1% isoamyl alcohol by volume was used as extracting solvent for ephedrine since the sensitivity is enhanced about 50% by the use of this solvent compared to benzene alone. Benzene alone was satisfactory for the other drugs as there was no appreciable difference in sensitivity or precision of assay between the two solvents.

For each drug, the dye concentration was chosen so that at the optimum concentration of drug the molar ratio of dye to drug was about 10 to 1. For most compounds at a fixed concentration, the observed absorbance is independent of the concentration of dye providing the molar ratio of dye to drug is greater than about 2 to 1. This is true for all the compounds examined in this study except ephedrine. With ephedrine,

TABLE I

ABSORBANCE OF STANDARD SOLUTIONS CONTAINING VARYING CONCENTRATIONS OF DRUG

Propoxyphene hydrochloride		*Cyclizine monohydrochloride*		*Chlorpheniramine hydrochloride*		*Diphenhydramine hydrochloride*		*Ephedrine sulfate*	
Concn. (μg/5 ml)	*Absorbance per 100 μg*	*Concn. (μg/5 ml)*	*Absorbance per 100 μg*	*Concn. (μg/5 ml)*	*Absorbance per 100 μg*	*Concn. (μg/5 ml)*	*Absorbance per 100 μg*	*Concn. (μg/5 ml)*	*Absorbance per 100 μg*
61.1	0.355	34.7	0.565	31.6	0.465	24.3	0.576	51.5	0.500
81.4	0.409	52.0	0.567	47.9	0.481	48.6	0.595	72.0	0.539
101.8	0.411	69.4	0.578	63.8	0.483	72.9	0.642	82.3	0.522
122.1	0.409	86.7	0.596	79.8	0.493	97.2	0.638	102.9	0.539
142.5	0.401	104.1	0.595	95.7	0.491	121.4	0.618	113.2	0.537
				111.7	0.490			133.8	0.520

* Cat. No. 15846, Wilkens-Anderson Co., Chicago.

the observed absorbance increases with increase in dye concentration. However, Beer's law is obeyed at any fixed concentration of dye under the experimental conditions described. Therefore it is essential to use the same solution of dye for each sequence of analyses in order to achieve accurate and precise results for ephedrine.

The major requirement of quantitative thin-layer chromatography, whether involving measurement of sample amount by spot area, by densitometry or by elution, is the application of a known amount of sample in a reproducible manner. FAIRBAIRN AND RELPH[4] recently have reported on the errors obtained in the production of the initial spot. In a review of the literature, they have pointed out typical examples where coefficients of variation are at least 5% or more, and coefficients of variation of 5–7% were obtained by those authors using the same technique on model systems. The error was shown to arise mainly from creep-back on the syringe needle during application of the solution to the chromatoplate. This difficulty could be largely overcome by the technique of forcible ejection of liquid from the syringe onto the plate, giving coefficients of variation of about 2%.

In the present study, creep-back on the syringe needle also was noted, especially with methanolic solutions. When using the hanging drop technique (*i.e.*, a small drop of liquid is produced on the tip of the needle and the chromatoplate touched to the drop and this sequence repeated until the desired volume is applied), only about 80% of the material reached the plate in some instances. The remainder stayed on the outside of the syringe needle. Consequently the technique was used of securely clamping the micrometer syringe in a vertical position, then mechanically raising the chromatoplate so that the needle point pierced the silica gel and rested against the glass plate. In this manner, solution from the syringe would flow directly on to the adsorbent layer. With most syringe needles, the length of the tip keeps the aperture above the chromatographic layer. At certain times, a droplet of liquid would form in the opening and reach a considerable size before touching the layer and being adsorbed. In such cases, creeping of the solvent up the outside of the needle would be significant (but occurring at irregular times). By reducing the length of the needle point by filing (and maintaining the bevel angle unchanged), the aperture of the syringe needle opened directly into the adsorbent layer. In this way, the solution would flow directly into the adsorbent without creep-back.

Following development of the chromatogram, spots were located under short-wave UV light, then the adsorbent containing each sample was sucked off the plate into the modified sintered glass funnel. The tip of tygon tubing served adequately to remove all the adsorbent of interest from the plate. Five milliliters of 1% (v/v) acid was found to be more than adequate for elution of each amine from the adsorbent. Additional solvent passed through the system gave a blank reading with the eluate. The volume of 5 ml of eluting solvent was convenient for use with the experimental conditions for the acid dye technique, *i.e.*, sample in 5 ml aqueous solution, 10 ml total of aqueous buffer and indicator dye solution, and extraction of the ion-pair into 10 ml of photometric solvent. The total volume of aqueous and organic solvent (25 ml) then allowed convenient mixing or extraction in a 42 ml centrifuge tube which could be used with a bench top centrifuge. It was noted that a higher sensitivity was achieved using 1% (v/v) HCl as eluting solvent compared with 0.1 *N* H_2SO_4. With propoxyphene, for example, the sensitivity was about 15% greater using 1% (v/v) HCl whereas with ephedrine the sensitivity was about 5% greater. Both eluting solvents resulted

in the same over-all recovery from the chromatogram. Therefore 1% (v/v) HCl was adopted as solvent of choice for elution.

Comparison of the absorbance obtained using 5 ml of eluate from a blank chromatogram against the absorbance from 5 ml of 1% (v/v) HCl showed no elution of material from the adsorbent layer which would interfere in the quantitative measurement. Nevertheless, a blank from the chromatogram was always used when analyzing samples recovered from the same plate. Thus the acid dye technique overcomes the problem of interference encountered when quantitation is by direct UV absorbance of the eluate. SPENCER AND BEGGS[5] in a detailed study of errors occurring in analysis by UV measurement found that fines eluted from silica gel gave a significant contribution to the over-all absorbance. This interference could be eliminated only by filtration through a 0.45 μ synthetic membrane filter. Likewise, MORRISON AND CHATTEN[6] observed that the amount of barbiturate eluted from a chromatoplate was grossly overestimated when measured by direct UV absorbance. The problem was overcome by aqueous elution followed by extraction of the mercury salt into chloroform and determination of the mercury by a dithizone procedure.

TABLE II

RECOVERY OF SAMPLE FROM CHROMATOPLATE

Compound spotted	*Operator*	*Micrograms spotted*	*% recovered*		*No. of Determinations*
			Av.	*S.D.*	
Chlorpheniramine maleate[a]	A	79.76	98.9	0.8	6
	A	64.60	97.1	0.8	6
Chlorpheniramine maleate[b]	A	79.76	98.0	0.4	6
	A	64.60	97.8	0.8	6
Cyclizine monohydrochloride[b]	A	85.90	96.7	0.8	5
	B	85.90	96.8	0.6	5
Diphenhydramine hydrochloride[b]	A	90.63	97.7	0.5	6
Ephedrine sulfate[b]	A	82.75	95.0	0.7	6
Propoxyphene hydrochloride[b]	A	128.14	96.1	1.0	5
	B	128.14	96.5	0.8	5

[a] Sample chromatographed just off origin with neutral developing solvent. Layer removed from both application and sample zone.

[b] Sample chromatographed in the ammoniacal solvent system. Layer removed from sample zone only.

Table II shows typical recoveries and reproducibility with the five amine drugs used in the study. The weight of drug applied was the amount theoretically delivered by the Agla syringe for each setting of 10 μl on the micrometer. No attempt was made to calibrate the syringe for absolute volume delivered, although the amount was within 3% of that found by dilution of the sample solution with a macropipet and measurement of the concentration. The recovery of sample from the chromatoplate was determined by comparison with the amount delivered directly from the micrometer

syringe into a centrifuge tube. For delivery, the aperture of the syringe needle was touched to the inside wall of the tube and 10 μl expelled in one continuous motion. In each analysis, the average absorbance from four such tubes was used for determination of recovery from the chromatoplate. The top entry in Table II shows a duplicate set of six recoveries obtained by chromatographing the sample just off the origin (using a neutral developing solvent) and removing the layer containing the sample as well as the application zone. The second entry for chlorpheniramine in Table II shows the recovery and reproducibility are essentially the same for samples before and after chromatography. Results for the other compounds in Table II show a similar degree of recovery and reproducibility. No appreciable difference occurred between operators using the same experimental technique. For all determinations, the average recovery amounted to 97.1%, with a coefficient of variation of 0.7%.

TABLE III

ANALYSIS OF TWO-COMPONENT MIXTURES OF AMINES[a]

Mixture No.	*Operator*	*Components*	*Micrograms spotted*[c]	*% recovered*			
				Spot 1	*Spot 2*	*Spot 3*	*Av.*
1	A	Diphenhydramine hydrochloride	78.86	102.0	103.4	101.4	102.3
		Ephedrine sulfate	109.91	98.6	97.7	99.5	98.6
1	B	Diphenhydramine hydrochloride	79.45	102.2	102.0	101.6	101.9
		Ephedrine sulfate	100.75	100.0	98.7	99.1	99.3
2	A	Diphenhydramine hydrochloride	76.39	102.3	100.4	—	101.4
		Chlorpheniramine maleate	100.91	98.5	100.4	98.8	99.2
2	B	Diphenhydramine hydrochloride	75.95	100.2	99.4	99.8	99.8
		Chlorpheniramine maleate	101.03	98.6	99.5	100.0	99.4
2[b]	—	Diphenhydramine hydrochloride	75.95	100.0	99.8	100.0	99.9
		Chlorpheniramine maleate	101.03	96.1	96.6	97.8	96.8
3	A	Chlorpheniramine maleate	101.84	100.8	102.0	101.4	101.4
		Ephedrine sulfate	100.32	100.6	101.3	101.1	101.0
3	B	Chlorpheniramine maleate	104.32	98.9	101.1	99.5	99.8
		Ephedrine sulfate	102.82	100.0	100.4	100.0	100.1

[a] Three volumes of sample solution spotted at separate locations and the recovery of each compared to the average of three reference standards on the same chromatoplate.
[b] Chromatoplate spotted and chromatographed, then left overnight before elution.
[c] Theoretical amount contained in each 10 μl of sample solution.

Table III, showing the results of analyses of several two-component mixtures of amines, demonstrates the accuracy and precision that may be achieved by the described experimental procedure. Results between operators were in good agreement, with the largest difference amounting to 1.6%. The average recovery for all spots (excluding 2[b]) was 100.3% with a coefficient of variation (CV) of 1.2. The average recovery for all spots by operator A was 100.6% (CV = 1.5) while that for operator B was 100.1% (CV = 1.1). Since the above data were calculated from the results of three separate sample spots compared to the average of three standards on the same chromatoplate, the true variability under conditions of assay will be greater. Nevertheless,

the combination of the acid dye technique with quantitative thin-layer chromatography offers a relatively rapid and precise means for the analysis of mixtures of amines.

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